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Bioactivity-guided isolation of anticancer compounds from *Euphorbia lathyris*

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1. Introduction

Based on the number of cases reported, cancer is considered as a fatal disease in the world.1,2 However, the method of cancer therapy is very limited. Current primary treatment for cancer is chemotherapy, but serious side effects have been reported.3 Thus, a continued effort for discovery of novel anticancer compounds is needed. *Euphorbia lathyris*, a potent and valuable source of traditional Chinese medicine (TCM) which has been used hydropsy, ascites, coprostasis, anuresis, amenorrhea, stasis, terminal schistosomiasis, scabies and snakebite.4 Previous studies of the anticancer activity of *Euphorbia lathyris* showed that bioactive components had mainly focused on water or alcohol extracts. Recently, numerous researches demonstrated that the ethyl acetate extracts of *Euphorbia lathyris* have more potent and broad-spectrum anticancer activity than its hot water and ethanol extracts.5 The anticancer constituents of the ethyl acetate extracts from *Euphorbia lathyris* were not investigated earlier. The aim of the present work was the isolation and identification of the anticancer compounds from the ethyl acetate extracts of *Euphorbia lathyris* using bioactivity-guided separation method.

Searching for natural bioactive anticancer compounds requires appropriate bioassays. Recently, a new technological advance which is an electronic real-time cell analyzer (RTCA) makes it possible to obtain real time data on changes in cell growth,
30 morphology and cell death induced by drugs. As the measurement is non-invasive and
labeled free, the system can continuously monitor cells from the time when they are
seeded. In addition, compared to MTT assay, RTCA provides precise information not
available with other technologies after the addition of chemical compounds are
recorded. The results are presented as a cellular index (CI), the more live cells that are
attached to the sensor surface, the higher the CI value.

In previous work, separation and purification of compounds from *Euphorbia*
*lathyris* mainly relied on traditional methods of separation and purification, such as
column chromatography, TLC, macroporous resin chromatography, and gel exclusion
chromatography. Nevertheless, because of successive extractions with solvents,
successive column isolation, low column efficiency, bad separation repeatability and
limited column resolution, leading these fractions obtained with low pharmacology
activity and poor purity. Taking these problems into consideration,
two-dimensional preparative HPLC, which involves coupling two independent
mechanisms of separation, is regarded as more selective and having more resolving
power than one-dimensional chromatography; it is, therefore, more useful for
separation of complex samples.

Two-dimensional RP–RPLC is a practical separation method and has been used
for isolation of compounds from pharmaceutical samples. Two-dimensional HILIC
coupled with RP liquid chromatography has been proved to be very useful for
separation of the components of *Taxus baccata* L. Although these preparative 2D
HPLC systems enable reasonable separation of compounds with a wide range of
polarity and can, to some extent, solve the problems of peak capacity and separation
selectivity, there are still specific deficiencies in comprehensive characterization of
weakly polar TCM extracts, because of limitations of the modes of separation and the
complexity of the samples. Thus, a specialized 2D HPLC method for preparative
separation of weakly polar extracts of natural products is urgently needed.

In addition, two-dimensional HPLC can be performed either off-line or on-line.
In this study, off-line 2D-LC analysis of TCMs is seemingly time-consuming method,
but it provides valuable information for the separation of active compounds in one-dimensional HPLC or even multi-dimensional HPLC analysis. Moreover, considering that the off-line mode has no restrictions on the screening of the bioactive components using RTCA, so the bioactive fractions eluted from the first dimension could be prepared and analyzed with enough time. Therefore, the off-line mode was selected to maximize resolving power of two dimensions.

The purpose of the work discussed in this paper was to develop a simple method, with *Euphorbia lathyris* example, based on the use of a novel nonaqueous 2D preparative high-performance liquid chromatography (PHPLC), the real-time cell analyzer (RTCA), and X-ray single crystal diffraction (XRSCD), for efficient isolation and identification of anticancer compounds.

2 Results and Discussion

2.1 Establishment of Bioactivity-guided Off-Line 2D Chromatography System

In bioassay-guided isolation, the steps of the procedure (separation, fractionation, and purification) are systematically followed by bioassay, i.e. the next step of the process is based on a bioassay result. For this reason, rapid, reliable and relatively simple biological detection is needed. RTCA, which enables rapid measurement of the dynamic biological response of living cells in the presence of compounds which have cell growth-inhibiting or promoting properties, meets these demands.

For the sake of constructing a specialized 2D HPLC preparative separation method, several columns were tested. In consideration of the weak polarity of the components in our samples, nonaqueous column seemed to be the only option for the preparative separation. Moreover, to achieve better separations, selection of mobile phases of appropriate composition is also very important.

The main objective of the first-dimensional preparation is to simplify complex samples into fractions. The bare silica column is the most traditional nonaqueous normal-phase column for separation of weakly polar compound. Mobile phases used in traditional normal phase chromatography consist of a non-polar solvent and small amounts of polar organic solvents. In this work, several polar solvents mixed with a
non-polar solvent (n-hexane) were investigated to test the results of NP separations. When UV detection is used, the choice of mobile phase is limited. Because of interference as a result of solvent absorption in the UV region, the combinations n-hexane–acetone and n-hexane–ethyl acetate did not provide good baseline separation. However, the results showed that a mixture of n-hexane and ethanol as binary mobile phase for NP chromatography has such advantages as wide polarity range, by changing the ratio of the two solvents, and relatively low environmental toxicity. Thus, bare silica columns with a nonaqueous mobile phase were selected for separation in the first dimension.

In normal phase (NP) systems, the following equation was found to adequately describe the retention model:

\[
\log k = \log k' - m' \log \phi \tag{1}
\]

where \( \phi \) was the volume fraction of the polar solvent, \( k' \) was the retention factor, and \( m' \) was the stoichiometric coefficient characterizing the number of the molecules of the strong solvent that were needed to displace one adsorbed molecule of the analyte.

There were three variables that affect the retention factor \( k \), which are \( k' \), \( m' \), and \( \phi \). When the analytes and mobile phase system (n-hexane/ethanol) were determined, the retention factor \( k' \) could be tested and the \( k' \) became a constant. On the basis of the condition that analytes and mobile phase system were both determined, if the concentration of the sample solution and loading volume were invariable, the number of the molecules of the strong solvent that were needed to displace one adsorbed molecule of the analyte could also be determined, which means that the variable \( m' \) became a constant. Hence, we defined, Eq. (1) could be simplified as:

\[
\log k = \log K_1 - M_1 \log \phi \tag{2}
\]

Here \( K_1 \) and \( M_1 \) were two constant parameters and \( \phi \) was the volume fraction of the ethanol.

The main objective of the second-dimensional preparation is to provide high-performance and rapid preparation for the fractions collected from the first-dimensional preparation. High-performance separation benefits from the
second-dimensional column whose retention mechanism is different as the first-dimensional column, it was possible to form an orthogonal separation system. Of all two-dimensional liquid chromatography methods, the coupling of normal phase liquid chromatography (NPLC) to reverse phase liquid chromatography (RPLC) holds most promise because of the orthogonal nature of the separation mechanisms. A typical mobile phase for RPLC includes water-miscible polar organic solvents such as acetonitrile with a small amount of water. Unfortunately, weakly polar samples are usually too strongly retain in RP LC and (or) are not sufficiently soluble in the aqueous mobile phases. HILIC is a complementary technique to both normal and reversed-phase chromatography for the separation of compounds. This problem can be solved by using Hydrophilic interaction chromatography (HILIC) amide column. However, amide column was previously usually used in aqueous HILIC mode. In nonaqueous chromatography, amide column was only used for analysis. No report on amide preparative column coupled with bare silica column used in nonaqueous 2D preparative chromatography could be found.

For HILIC mode, the retention model can be described by the following equation:

\[ \log k = \alpha + m_1\varphi_1 - m_2\log\varphi_1 \]  

The \( \alpha \) is an empirical constant. The term \( m_1\varphi_1 \) was related to the interaction between solutes and solvents (RP effect). While \( m_2\log\varphi_1 \) was related to the direct analyte–stationary phase interaction (NP effect). Fig. S1 showed an example illustrating the effect of the mobile phase composition on the retention factors of caffeic acids. The U-shape curves got by increasing the concentration of water were similar, which provides evidence for the fact both HILIC and RP mode share the same separation mechanism. However, as the concentration of acetonitrile increasing, strong deviations start to take place. That showed the separation effects of the two different modes were opposite. This phenomenon goes for normal phase liquid chromatography. Therefore, when a double organic solvent system was employed on an amide column, the interaction energy between solutes and solvents became so
weak. Then, the term $m_1\phi_1$ could be approximately considered as a small and nonignorable constant. Then, the variable $\phi_1$ became $\phi$ that was the volume fraction of the ethanol. So the NP term $-m_2\log\phi_1$ became $-M_2\log\phi$. Eq. (3) could be finally simplified as the following equation:

$$\log k = K_2 - M_2\log\phi$$

Among which $K_2$ was a constant, and $M_2$ was a constant that was a new coefficient related to analyte–stationary phase interaction.

The two different parameters $M_1$ and $M_2$ indicated that the sensitivities of the bare silica column and nonaqueous amide column against mobile phase changing might be different. Additionally, the retention characters of the two columns might be different too, due to their different intercepts $K_1$ and $K_2$. On the basis of the above differences between the two columns, they would have selectivity characters that were distinct enough to form an orthogonal 2D chromatography system.

### 2.2 First Dimension Preparative HPLC

The first dimensional preparation was carried on Agela bare silica prep column. The sample injection volume for each run was 150 mL. One whole preparation procedure took 65 min including 10 min for column balance, 45 min for preparation and 10 min for column washing. The retention time of the components differed significantly because of the complex composition of the extracts. Isocratic elution fails to achieve satisfying separations. Hence, gradient elution was necessary in first dimensional preparation. Linear gradient provided better separation performance for the constituent with different retention times. Good separation performance can be obtained in analytical HPLC (Fig. 1a). When using the same chromatographic conditions in preparative HPLC, the similar retention times was obtained as analytical HPLC. Finally, analytical elution methods were used for the preparation. Preparation of the total 200 g extracts required 14 injections over 16 h. According to UV absorption intensity, the fractions were collected to reduce the complexity of each fraction as much as possible. The cross in each fraction has been minimized because of good separation repeatability. As shown in (Fig. 1b), eleven fractions were
collected in the first dimensional preparation. All fractions were analyzed by the
RTCA using A549 and HEPG2 cells to evaluate their anticancer activities. As the
results presented in Fig. 4 and Fig. 5, fractions 3, 5 and 6 showed higher bioactivity
than the other fractions. Thus, they were worthy of further separation and purification
by the second dimensional HPLC.

2.3 Analysis on Fractions 3, 5 and 6 on amide column and bare silica column

Fractions 3, 5 and 6 were analyzed on the YMC amide column to compare the
separation selectivity with that on the Agela bare silica column. Good orthogonality
was significant to achieve efficient preparation of compounds with high purity from
the samples. For example, Fr. 3 was roughly a single peak on bare silica column and it
might be regarded as a single compound in traditional technique where HPLC
preparation was commonly used in the final step to yield pure compounds. In fact, on
amide column, there were two peaks (Fig. 3). Moreover, Fr. 5-4 to Fr. 5-6 could be
clearly recognized on amide column. However, on bare silica column their retention
times were so close that it was impossible to obtain satisfactory resolution. For the
coevaluted compounds samples such as Fr. 6-7 (Fig. 3), it may be because of the
presence of a variety of compounds of the same polarity and a relatively low
concentration of minor compounds. But, multiple compounds with high purity can be
prepared on the amide column. In view of its higher separation efficiency, better
resolution and peak shape, the amide column was chosen as the second dimensional
column for further separation. It not only improved the separation efficiency but also
made a remarkable contribution to the purity of the compounds collected.

2.4 Preparative HPLC in the Second Dimension

In 2D LC, the second dimension LC is important in achieving higher separation
efficiency. The second-dimensional separation was performed on an amide column. In
order to purify the single compounds effectively, the separation condition was
optimized for fractions 3, 5 and 6. In this study, preparative scale conditions were
optimized in an analytical scale. For fraction 3, it was found that second-dimensional
separation should be performed under isocratic conditions to avoid time-consuming
reequilibration required for reconditioning in gradient elution. However, for fractions 5 and 6, simple isocratic method was not enough. When using isocratic elution, high concentration of ethanol at the beginning results in shorter retention times, even cause changes of peaks order. Thus, gradient elution was necessary preparative separation in the second dimension. After minor modification, the optimized conditions used for analytical chromatography were used for preparative scale separations. The analytical chromatogram in Fig. 2b showed similar patterns to the preparative chromatograms in Fig. 3, which demonstrated the feasibility of the transformation from an analytical scale to a preparative one. Moreover, it was also worthy of note that heart-cutting was used as the repeated separation strategy to ensure the purity of the compounds. Good separation resulted in several compounds were obtained and dried through rotary evaporation at 60 °C in vacuum.

In bioassay experiments, each fraction was tested for *in vitro* anti-lung cancer and anti-liver cancer activity, respectively (Fig. 4c - 4f and Fig. 5c- 5f). Among all the fractions, the seven fractions (Fr. 3-4, 36 mg), (Fr. 3-5, 38 mg), (Fr. 5-4, 60.2 mg), (Fr. 5-6, 10.3 mg), (Fr. 6-3, 15.1 mg), (Fr. 6-7, 18.2 mg) and (Fr. 6-11, 16.8 mg) had different degree of growth inhibition effect against A549 and HEPG2 cells at a concentration of 50 µg·mL⁻¹. Among all fractions, Fr. 3-5 and Fr. 6-7 showed significant anticancer activity against A549 and HEPG2 cells. Fr. 3-4, 5-4, 5-6 and 6-11 were inapparent active against HEPG2 cells. However, when tested in the A549 cells, the four fractions showed different degree of anticancer activity. Fr. 6-3 exhibited a good inhibition of cancer cells growth activity.

The purity of these compounds was checked by HPLC, which is shown in Fig. 6 and all compounds were more than 95 % purity. As described above, the seven fractions were of high purity, which require structural identification.

In a word, the first-dimensional preparation efficiently simplified the sample and improved the separation on the second-dimensional preparation, so that multiple compounds with high purity can be prepared at the second-dimensional preparation.

### 2.5 Structural identification of bioactive compounds
Compounds 1-7 were identified by 1D ($^1$H (400 MHz), $^{13}$C (100 MHz) and DEPT-135) and 2D NMR spectroscopy. Complete assignment of all the proton and carbon data was based on $^1$H–$^1$H COSY, HSQC, and HMBC data. The results are listed in ESI Table S1 and Table S2. Comparison of the $^1$H and $^{13}$C NMR spectra of these compounds with the previous reports characterized their structures as *Euphorbia* Factor L2 (1), *Euphorbia* Factor L1 (2), Glyceryl monoooleate (3), *Euphorbia* Factor L8 (4), *Euphorbia* Factor L3 (5), *Euphorbia* Factor L9 (6) and Esculetin (7). The structures of these compounds were shown in Fig. 7.\textsuperscript{25-27}

Compound 2 was *Euphorbia* Factor L1, a compound that had been previously reported.\textsuperscript{28} Since the $^1$H and $^{13}$C spectra showed severely overlapping signals in the high field region, and given that the chemical shift values slightly differed from reported data, we confirmed the structure with the aid of X-ray diffraction analysis.

According to X-ray single-crystal diffraction (XRSCD) data, chiral centers C-2, C-3, C-6, C-9, and C-11 were assigned the S configuration and C-4, C-5, and C-15 were assigned the R configuration. The structure of compound 2 determined by XRSCD, with the atom numbering scheme, is shown in Fig. 8. Selected bond distances and angles are listed in ESI Table S4 and Table S5. On the basis of these data the absolute configuration of compound 2 was confirmed.

### 3. Experimental

#### 3.1 Apparatus

An industrial prep-HPLC system consists of two prep-HPLC pumps, a UV detector and a HPLC workstation (Jiangsu Hanbon Sci. & Tech. CO., Ltd, Huaian, China). The semi-preparation HPLC system was carried on Agela CHEETAH MP 100 system, which consists of two binary gradient pumps, a UV detector, a sample collector and an Agela LC software (Bonna-Agela Technologies, Tianjin, China). Chromatographic analysis was performed with a Hitachi HPLC system comprising two Hitachi L2130 pumps, a Hitachi L2400 UV diode-array detector, Hitachi L2200 autosampler, column thermostat and a Hitachi Lachrom Elite HPLC workstation (Hitachi, Tokyo, Japan). The NMR spectrum was measured by a Bruker DXR 400 NMR spectrometer with
CDCl₃ and dimethyl sulfoxide (DMSO) as solvents (Bruker AVANCE III; Bruker, Karlsruhe, Germany). The iCELLigence RTCA system was composed of three main components: an iCELLigence RTCA analyzer, RTCA control unit (iPad with integrated software) and a disposable E-Plate L8 (ACEA Biosciences, USA).

### 3.2 Reagents

The Industrial and prep-HPLC grade ethanol, n-hexane and ethyl acetate were purchased from Concord Technology Co. Ltd. (Tianjin, China). HPLC grade solvents were purchased from Honeywell (USA). DMSO was purchased from the Sino-American Biotechnology Company of Beijing (Beijing, China). Minimum essential medium (MEM) and F-12K medium was purchased from Life Technologies (USA).

### 3.3 Sample Preparation

*Euophorbia lathyris* was purchased from Anguo medicinal materials market (Anguo, Hebei Province, China). The plant material was identified by Professor Lijun Zhou (Tianjin University). Five kilograms *Euophorbia lathyris* powders were obtained by ultrafine comminution (SQW-25; San Qing Technology Company, Jinan, China) at low temperature. Then, the powder was extracted three times by 40 L ethyl acetate for 3 h. After centrifugation, the supernatant was combined and concentrated by rotary evaporation (RE-52AA; Yarong Biochemical Instruments, Shanghai, China) at 60 °C in a vacuum. The ethyl acetate extract was dissolved in a solvent of n-hexane–ethanol (70:30, v/v). The solution was filtered through 0.45 μm membranes. The final concentration of sample solution was 200 mg·mL⁻¹.

### 3.4 Analytical HPLC

#### 3.4.1 1D-HPLC analysis

The ethyl acetate extracts were chromatographed on an Agela bare silica column (4.6 × 250 mm, 10 μm, 100 Å). The injection volume was 10 μL. The mobile phase was a gradient prepared from n-hexane (solvent A) and ethanol (solvent B); the gradient program (v/v) was: 5 min 100 % solvent A, 40 min 0-13 % solvent B, 10 min 100 % solvent B; the flow rate was 0.6 mL·min⁻¹. HPLC analysis was performed at room temperature and monitored at 210 nm.
3.4.2 2D-HPLC analysis Analysis of the bioactive fraction was performed at room temperature by HPLC on YMC amide and Agela bare silica column (4.6 × 250 mm, 10 μm, 100 Å) with n-hexane (solvent A) and ethanol (solvent B). Different elution conditions were adopted to analysis fractions collected from the first dimensional separation. The chromatographic conditions for fraction 3 was 5 % solvent B for 65 min, for fraction 5 was 0-20 % solvent B for 65 min, for fraction 6 was 0-25 % solvent B for 65 min. The injection volume was respectively 10 μL. The flow rate was 0.6 mL·min⁻¹. The chromatographic data was collected at 210 nm.

3.5 Preparative HPLC

3.5.1 1D-HPLC Preparation The first dimensional preparation was performed on an Agela bare silica prep column (150 × 250 mm, 10 μm, 100 Å). The mobile phase A was n-hexane and mobile phase B was ethanol. The linear gradient elution steps were as followed: 0-5 min 100 % A, 5-45 min 0-13 % B, 45-55 min 100 % B. The flow rate was 600 mL·min⁻¹. The sample concentration was 200 mg·mL⁻¹. The injection volume was 150 mL. Chromatography was recorded at 210 nm. Each fraction was dried under vacuum in a desiccator and stored under dry conditions for further separation and purification.

3.5.2 2D-HPLC Preparation The second dimensional purification was performed on a YMC amide column (10 × 250 mm, 10 μm, 100 Å). The mobile phase A was n-hexane and mobile phase B was ethanol. Fractions collected from the first dimensional separation were further purified on amide column to obtain single compounds. Different elution conditions were adopted to separate fractions collected from the first dimensional preparation. The elution procedure for fraction 3 was 5 % B for 65 min, for fraction 5 was 0-20 % B for 65 min, for fraction 6 was 0-25 % B for 65 min. The sample concentration was 200 mg·mL⁻¹. The injection volume was 50 μL. The flow rate was 3 mL·min⁻¹. The chromatographic data were collected at 210 nm.

3.6 Bioassays

The anticancer effect of fractions was evaluated by use of the iCELLigence RTCA against A549 cells and HEPG2 cells. The iCELLigence RTCA system consists of 8
well E-Plates (E-Plates 8) engaged on stations where the cell index (CI) reading was recorded simultaneously. 150 μL MEM medium was added to each well of the E-plates 8 for A549 cells and 150 μL F-12K medium for the HEPG2 cells, respectively. Then, the plates were placed in the iCELLigence RTCA system and scanned for 2 min at 37 °C and 5 % CO₂. The plates were incubated for 30 min to obtain a stable noise signal. A549 cells (2 × 10⁴/well) and HEPG2 cells (4 × 10⁴/well) were cultured in each well of the E-plates 8 respectively. Samples containing untreated A549 and HEPG2 cells served as a control for the assay. Following exposure to 50 μg·mL⁻¹ samples, the cell index (CI) reading frequency was once per minute until completion of experiment. The experiment was conducted for 48 h.

3.7 Purity Analysis of Bioactive Compounds

Purity test of bioactive compounds were performed on a YMC diol column (4.6 × 250 mm, 5 μm, 100 Å). The mobile phase A was n-hexane and mobile phase B was ethanol. The flow rate was 1 mL·min⁻¹. The injection volume was 10 μL. The elution procedure for Fr. 3-4 and Fr. 3-5 was 2 % - 6 % B for 20 min, Fr. 5-4 and Fr. 5-6 was 0 % - 20 % B for 20 min, for Fr. 6-3 was 7 % B for 20 min, Fr. 6-7 was 0 % - 15 % B for 25 min and Fr. 6-11 was 0 % - 25 % B for 25 min, respectively. Chromatographic data were collected at 210 nm.

3.8 NMR Spectrometry

Structures of anticancer compounds were identified by one and two dimensional nuclear magnetic resonance spectroscopy (1D and 2D NMR). NMR experiments were performed with a Bruker AVANCE III NMR (400 MHz) spectrometer. Samples were dissolved in CDCl₃ and DMSO, and chemical shifts were reported relative to the resonance of TMS at δ = 0 ppm as reference. Solution volume was concentrated to 400 μL and transferred to a 5 mm NMR test tube for analysis.

3.9 X-ray single Crystal Diffraction

Single crystal of compound 2 was performed by slow evaporation, at 4 °C, of a solution in n-hexane–tetrahydrofuran (1:1). A colorless single crystal (0.25 mm × 0.20 mm × 0.15 mm) was selected and used for the X-ray diffraction experiment. X-ray
Diffraction data were collected on a Rigaku Raxis Rapid IP detector equipped with a graphite-monochromatic Mo-Kα radiation (k = 0.71073 Å) at 293(2) K. The orientation matrix and unit cell parameters were obtained from the setting angles of 25-centered reflection. The crystals were orthorhombic, space group P2₁2₁2₁ with a = 8.2474(16) Å, b = 2.543(3)(16) Å, c = 29.019(6) Å, α = 90.00, β = 90.00, γ = 90.00, V = 3001.9(10) Å³, Z = 4, Dcalc = 1.233 mg·m⁻³, and μ = 0.087 mm⁻¹. The diffraction intensities were collected by ω scanning (3.04-25.50). A total of 24,238/5,565 reflections were collected (-8 ≤ h ≤ 9, -15 ≤ k ≤ 15, -34 ≤ l ≤ 35). The crystallographic data and refinement details are summarized in Table S3. The crystal structure was solved by direct methods with SHELX-97 program and refined by full-matrix least-squares method.

4 Conclusions

On the basis of bioactivity-guided off-line isolation by 2D chromatography, seven anticancer compounds were isolated from an ethyl acetate extract of Euphorbia lathyris. A bare silica preparative column was used to obtain fractions from preparative separation in the first dimension. An amide preparative column used in nonaqueous mobile phases was employed to prepare compounds of high purity in the second dimensional preparation. Three fractions obtained from separation in the first dimension were analyzed on bare silica and amide columns. The results showed the good orthogonality of the 2D preparative separation. Benefitting from good orthogonality and optimized collection, seven bioactive compounds with > 95% purity were obtained by 2D preparative chromatography.

A novel bioassay-guided separation system was established for the separation of complex weakly polar bioactive samples. In this system, a HILIC column used with a nonaqueous mobile phase had good orthogonality when coupled with a traditional normal-phase column. RTCA is a useful tool for study of TCM with anticancer potential and for monitoring the bioactivity of fractionated components and pure compounds, which can be identified by the use of intelligent biosensor techniques.

We believe this novel bioassay-guided off-line 2D HPLC method is extremely
useful for preparative separation of weakly polar compounds and could lead to the
discovery of more useful natural anticancer compounds.

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Appendices
Figure 1. (a) One-dimensional analysis chromatogram of the ethyl acetate extract of *Euphorbia lathyris* on Agela bare silica column (4.6 × 250 mm, 10 μm, 100 Å). (b) One-dimensional preparation chromatogram of the ethyl acetate extract of *Euphorbia lathyris* on Agela bare silica column (150 × 250 mm, 10 μm, 100 Å).

Figure 2. (a) Second-dimension analysis chromatogram of Fr. 3, Fr. 5 and Fr. 6 on Agela bare silica column (4.6 × 250 mm, 10 μm, 100 Å). (b) Second-dimension analysis chromatogram of Fr. 3, Fr. 5 and Fr. 6 on YMC amide column (4.6 × 250 mm, 10 μm, 100 Å).
Figure 3. Second dimensional preparation chromatogram of Fr. 3, Fr. 5 and Fr. 6 on YMC amide column (10 mm × 250 mm, 10 μm, 100 Å).

Figure 4. Effect of fractions on the viability of A549 cells, determined by use of the...
iCELLigence RTCA system. Human lung cancer A549 cells at a density of 20,000 cells/well in E-Plate L8 were observed for 48 h. (a) Anti-lung cancer effect of Fractions 1–5. (b) Anti-lung cancer effect of Fractions 6–11. (c) Anti-lung cancer effect of fractions 3-1–3-6. (d) Anti-lung cancer effect of fractions 5-1–5-7. (e) Anti-lung cancer effect of fractions 6-1–6-5. (f) Anti-lung cancer effect of fractions 6-6–6-11.

**Figure 5.** Effect of fractions on the viability of HEPG2 cells, determined by use of the iCELLigence RTCA system. Human liver cancer HEPG2 cells at a density of 40,000 cells/well in E-Plate L8 were observed for 48 h. (a) Anti-liver cancer effect of Fractions 1–5. (b) Anti-liver cancer effect of Fractions 6–11. (c) Anti-liver cancer effect of fractions 3-1–3-6. (d) Anti-liver cancer effect of fractions 5-1–5-7. (e) Anti-liver cancer effect of fractions 6-1–6-5. (f) Anti-liver cancer effect of fractions 6-6–6-11.
Figure 6. The purity of isolated compounds was tested by HPLC using a YMC diol column (4.6 × 250 mm, 5 μm, 100 Å).
**Figure 7.** Chemical structures of bioactive compounds.

1. *Euphorbia* Factor L2 $R_1 = R_2 =$ benzoyl
2. *Euphorbia* Factor L1
3. Glyceryl monooleate
4. *Euphorbia* Factor L8 $R_1 =$ ONic
5. *Euphorbia* Factor L3 $R_1 =$ benzoyl
6. *Euphorbia* Factor L9 $R_1 =$ benzoyl $R_2 =$ ONic
7. Esculetin

**Figure 8.** X-ray single-crystal diffraction structural drawing with displacement ellipsoids showing the perspective view of the molecular conformation of compound 2, with the atom numbering scheme.

**Supplementary**
Table S1. $^{13}$C NMR spectroscopic data for compounds 1-6 in CDCl$_3$, compound 7 in DMSO (100 MHz)

Table S2. $^1$H NMR data for compounds 1-6 in CDCl$_3$, compound 7 in DMSO (400 MHz)

Table S3. Crystal data and structure refinement for compound 2

Table S4. Selected Bond Lengths (Å) for compound 2

Table S5. Selected Bond Angles (°) for compound 2

Figure S1. U-shape plots of the retention factors of caffeic acids, k, versus the volume fraction of water, φ (water)
Bioactivity-guided isolation of anticancer compounds from Euphorbia lathyris
39x27mm (300 x 300 DPI)